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Human recombinant alpha-parvalbumin and nine mutants with individually inactivated calcium- and magnesium-binding sites: biochemical and immunological properties

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Abstract

Human recombinant α-parvalbumin (PV_{wt}) and nine mutant proteins, containing inactivating substitutions at positions essential for Ca^{2+} binding in the CD Ca^{2+}-binding site (PV_{E62V}, PV_{D51A}, $PV_{D51A,62V}$), the EF site (PV_{E101V}, PV_{D90A}, $PV_{D90A,E101V}$) or in both ($PV_{E62V,E101V}$, $PV_{D51A,D90A}$, $PV_{D51A,E62V,D90A,E101V}$), were expressed and purified. Flow dialysis revealed that PV_{wt} binds 2 Ca^{2+} with equal K'_{Ca}, of 2.3×10^7 M^{-1} and that Mg^{2+} competes with a $K'_{Mg.compet.}$ of 4.9×10^3 M^{-1}. The three mutants with an inactivated CD site bind 1 Ca^{2+} with K'_{Ca}, of 2.0 to 2.3×10^7 M^{-1} and $K'_{Mg.compet.}$ of 3.4 to 4.6×10^3 M^{-1}, i.e. very similar to those of PV_{wt}. The mutants with an inactivated EF site bind 1 Ca^{2+} with K'_{Ca} values of 7.9×10^6, 4.5×10^6 and 3.6×10^6 M^{-1} for PV_{D91A}, PV_{E102V} and $PV_{E101V,D91A}$, respectively. The $K'_{Mg.compet.}$ values of these mutants are about 4-times lower than in PV_{wt}. The three mutants with both sites inactivated bind neither Ca^{2+} nor Mg^{2+}. After excitation at 259 nm, human PV, which contains neither Tyr nor Trp, shows maximal fluorescence emission at 283 nm. Binding of either Ca^{2+} or Mg^{2+} to PV_{wt} or to mutants with an inactivated EF site lead to a 1.8-fold decrease in fluorescence intensity, whereas the mutants with an inactivated CD show only a very slight decrease upon binding of Ca^{2+} or Mg^{2+}. Specific antibodies against human α-parvalbumin were raised in rabbits. Their reactivity was tested against the mutant proteins, and their potential value for location and functional studies was investigated.

Keywords: Calcium binding protein; α-Parvalbumin; EF-hand; Mutagenesis; Antibody; (Human)

1. Introduction

Parvalbumins (PV) belong to the large family of Ca^{2+}-binding proteins characterized by the EF-hand structural motif [1,2]. In vertebrates, two isoforms (α and β) are expressed, differing by several biochemical properties and their chromosomal location [3,4]. Furthermore, species differ in their number of isoforms, tissue expression, and cellular localization. For example, α-PV is highly expressed in the fast-twitch skeletal muscles of mice and rats [5] acting there as a muscle relaxation factor [1,6,7]. In the corresponding muscles of man, however, α-PV is expressed at a very low level [8]. Similarly, β-PV (also named oncomodulin) is highly expressed in rat tumors, but not in human neoplasms [9]. In man, β-PV is found in the cytotrophoblasts of the placenta [10]. This demonstrates that previous results on α- and β-PVs from various animal species are difficult to transfer to functional and clinical studies in humans.

One of our research goals is to study the function of α-PV in neurons of the central nervous system of man and try to explain its altered expression and cellular distribution in human neurodegenerative disorders [11]. Those studies have been inconsistent in the past because of a lack of specific human probes (cDNAs, proteins and antibodies) for cell transfection experiments and localization studies.

As a first approach, we expressed and purified human recombinant α-PV and nine mutants and studied the con-

Abbreviations: PV, α-parvalbumin; PAGE, polyacrylamide gel electrophoresis; ESI, electrospray ionisation; MS, mass spectrometry; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

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sequences on the properties of site-inactivation in human recombinant α-PV. Usually, inactivation of an EF-hand site is carried out by mutating the critical residue Asp in the +X position and/or Glu in −Z. Here, nine mutants with different combinations were constructed and the effect of the mode of inactivation on Ca^{2+}-binding properties was examined. In addition, we raised for the first time antibodies against human α-PV and tested its reactivity against the mutant proteins for future applications in functional studies.

2. Materials and methods

2.1. Mutations of human parvalbumin cDNA

Site-directed mutagenesis was carried out in order to modify the human PV cDNA [8] (GenBank/EMBL accession number X63070). One or both of the functional Ca^{2+}-binding sites (CD- or EF-domain) were inactivated by replacing either the first or last, or both, amino acids, Asp and Glu, in the Ca^{2+}-binding loops by the two non-polar amino acids, Ala and Val, respectively. The resulting mutant proteins are PV_{E101V}, PV_{D90A}, and $PV_{D90A,E101V}$ (collectively called PV_{-EF}), PV_{E62V}, PV_{D51A}, and $PV_{D51A,62V}$ (collectively called PV_{-CD}), $PV_{E62V,E101V}$, $PV_{D51A,D90A}$, and $PV_{D51A,E62V,D90A,E101V}$ (collectively called $PV_{-CD/-EF}$). All site directed mutagenesis experiments were performed as described [12] and confirmed by DNA sequence analysis [13]. The primers used to introduce mutations in the Ca^{2+}-binding sites were: PVD51A 5′-TTTTGTCCTTGGCCAGCATGT-3′, PVE62V 5′-TGAATCCCAGCACATCCTCCT-3′, PVD90A 5′-CCCATCTTTGGCTCCAGCAGC-3′, and PVE101V 5′-AGTGGAGAATACGTCAACCCC-3′.

2.2. Construction of expression vectors

The wild-type and nine mutant hPV fragments were cloned into the bacterial expression vector pGEMEX-2 (Promega, Madison, WI) where the *Nde*I cleavage site at position 3259 has been deleted. The PV mutant open reading frames were amplified by PCR using 5′ and 3′ flanking primers PVNDE 5′-AGTCATATGTCGATGACAGACTTG-3′ and PVHIND 5′-CTGAAAGCTTCAGAGAGGTGGAAGACCAGG-3′ in order to clone in the *Nde*I and *Hin*dIII cleavage site of pGEMEX-2. The constructs were confirmed by DNA sequencing.

2.3. Expression and purification of mutant proteins

Bacteria from *E. coli* strain BL21(DE3)pLysE carrying the expression plasmid were grown in Luria–Bertani medium containing 100 mg/l ampicillin, 10 mg/l chloramphenicol, and 2 mM $MgCl_2$. The culture was grown to an optical density of 0.4 at 600 nm, then 0.5 mM IPTG was added for another 4–5 h. Bacteria were harvested and resuspended in 10 mM Tris (pH 8.5) 0.1 mM PMSF. After sonication the cell lysate was frozen at −20°C. The thawed lysate was heated to 80°C for 10 min, cooled on ice, and centrifuged at 27 000 × *g* for 30 min at 4°C. The cleared lysate was adjusted to pH 8.5 and applied to a 1.6 × 6 cm Q Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden) equilibrated in the resuspension buffer. After washing the column by 20 bed volumes of 10 mM Tris (pH 8.5), the protein was eluted by addition of 50 mM NaCl to the washing buffer. Homogenous protein-containing fractions were identified by SDS-PAGE [14] and Coomassie brilliant blue staining.

2.4. Gel electrophoresis and mass spectrometry

SDS-PAGE (15%) under reducing conditions was performed as described [8]. IEF was carried out under denaturing conditions [15]. The isoelectric point of the proteins was calculated by the Wisconsin Software Package, Version 8 (Genetics Computer Group, Madison, WI, USA).

ESI-mass spectrometry and amino acid analysis of purified proteins was performed as described [16].

2.5. Protein and metal ion determination and metal removal

The proteins were precipitated with 3% trichloroacetic acid and then passed through a 40 × 1 cm Sephadex G-25 column equilibrated in Ca^{2+}-free 50 mM Tris-HCl (pH 7.5), 150 mM KCl (buffer A). Total Ca^{2+} and Mg^{2+} concentrations were determined with a Perkin-Elmer instrument 2380 atomic absorption spectrophotometer as previously described [17,18]. The protein concentration was determined from the ultraviolet absorption spectrum using a molar extinction coefficient at 259 nm of 2500 M^{-1} cm^{-1}.

2.6. Cation binding studies

Ca^{2+} binding was measured at 25°C by the flow dialysis method on 30–50 μM protein solutions, containing various concentrations of $MgCl_2$. Treatment of the raw data was as described by Cox et al. [19]; refined analysis of the Mg^{2+} effects was done as described by Pauls et al. [17]. We estimate that the accuracy on the binding constants obtained from flow dialysis experiments is around 80%.

2.7. Spectrofluorimetry

Emission fluorescence spectra were taken with a Perkin-Elmer LS-5B spectrofluorimeter on metal-free solutions of 50 μM mutant PVs in buffer A at room temperature with excitation wavelength at 259 nm and slits of 5 nm. 2 mM $MgCl_2$ and 1 mM $CaCl_2$ were added subse-

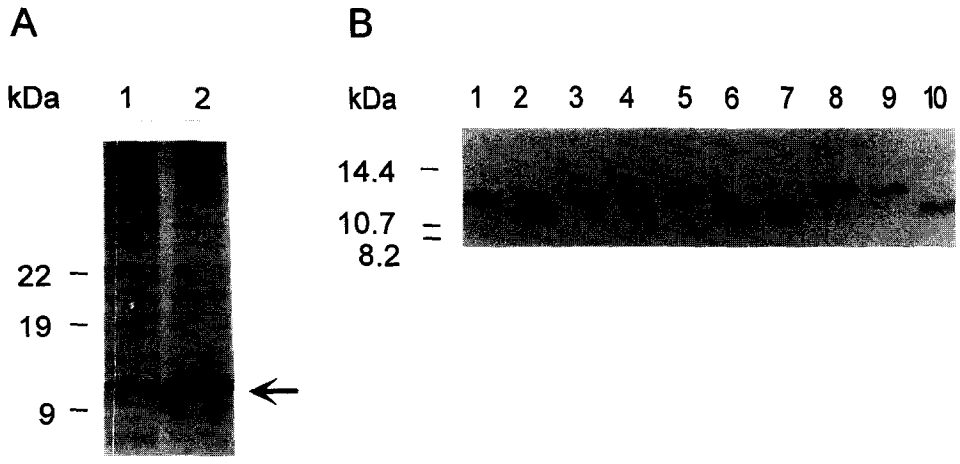

Fig. 1. Purification of the recombinant α-PV_{wt} and of the nine mutants. (A) SDS-PAGE of recombinant hPV_{wt} with 100 μl of bacterial culture before (lane 1) and after (lane 2) induction with IPTG. (B) Electrophoretic mobility of PV_{wt} and nine mutants in the presence of Ca^{2+}. 2 μg of each protein was applied. PV_{wt} (lane 1); PV_{E101V} (lane 2); PV_{D90A} (lane 3); PV_{E62V} (lane 4); PV_{D51A} (lane 5); $PV_{D90A,E101V}$ (lane 6); $PV_{E62V,E101V}$ (lane 7); $PV_{D51A,D90A}$ (lane 8); $PV_{D51A,E62V}$ (lane 9); $PV_{D51A,E62V,D90A,E101V}$ (lane 10). Proteins were visualized with Coomassie brilliant blue.

quently to obtain the Mg^{2+} and Ca^{2+} form, respectively. Alternatively, guanidine hydrochloride up to 4 M was added to obtain the fully denatured form.

2.8. Antibodies and Western blot analysis

Polyclonal antisera against human recombinant α-PV were raised in rabbits. Animals were immunized with 1 mg of protein in Freund's complete adjuvant and then boosted five times at 3 week intervals with 0.1 mg of protein in incomplete Freund's adjuvant. For Western blotting the two antisera pca63 and pcaF6 were diluted 1:5000. The specificity of both sera was identical. The monoclonal antibody against carp muscle PV, mca 235 [5,20] was diluted 1:2000. Alkaline phosphatase conjugated secondary antibodies were purchased from Promega (Madison, WI). For Western blot analysis proteins were separated on a 15% PAGE and blotted onto a Nytran membrane. Human cerebellum was homogenized in 10 mM Tris (pH 8.0), 1 mM EDTA, 0.5 mM PMSF, centrifuged at 15000 × *g* at 4°C for 30 min. The cleared supernatant was used for Western blot analysis.

3. Results

3.1. Purification and electrophoretic characterization of recombinant α-parvalbumin and nine mutants

Each of the mutant proteins contained substitutions in the Ca^{2+}-binding loops (see Section 2). High expression of hPV_{wt} and of the nine mutant proteins was observed in *E. coli* strain BL21(DE3)pLysE by addition of $MgCl_2$ to the culture medium. As an example, the expression of PV_{wt} is illustrated in Fig. 1. A bacterial culture extract before (Fig. 1A, lane 1) and after (lane 2) induction with IPTG was subjected to SDS-PAGE. After induction, approx. 40% of total protein represent parvalbumin.

Purification of PV_{wt} and nine mutant proteins was achieved by a heat step followed by ion exchange chro-

Table 1
Biochemical properties of human PV_{wt} and nine mutants

Protein	Isoelectric point		Molecular weight	
	calculated	measured	calculated	measured
PV_{wt}	4.84	5.34 ± 0.08	11927.44	11927.27 ± 1.79
PV_{E101V}	5.00	5.46 ± 0.10	11897.46	11898.64 ± 1.21
PV_{D90A}	5.02	5.47 ± 0.10	11883.43	11883.64 ± 0.98
PV_{E62V}	5.00	5.46 ± 0.10	11897.46	11897.32 ± 0.73
PV_{D51A}	5.02	5.49 ± 0.10	11883.43	11883.19 ± 1.43
$PV_{D90A,E101V}$	5.28	5.67 ± 0.06	11853.45	11853.58 ± 1.00
$PV_{E62V,E101V}$	5.26	5.64 ± 0.06	11867.48	11867.82 ± 1.23
$PV_{D51A,D90A}$	5.29	5.69 ± 0.06	11839.42	11839.39 ± 0.95
$PV_{D51A,E62V}$	5.28	5.67 ± 0.06	11853.45	11853.80 ± 1.41
$PV_{D51A,E62V,D90A,E101V}$	6.54	6.32 ± 0.12	11779.46	11779.57 ± 0.95

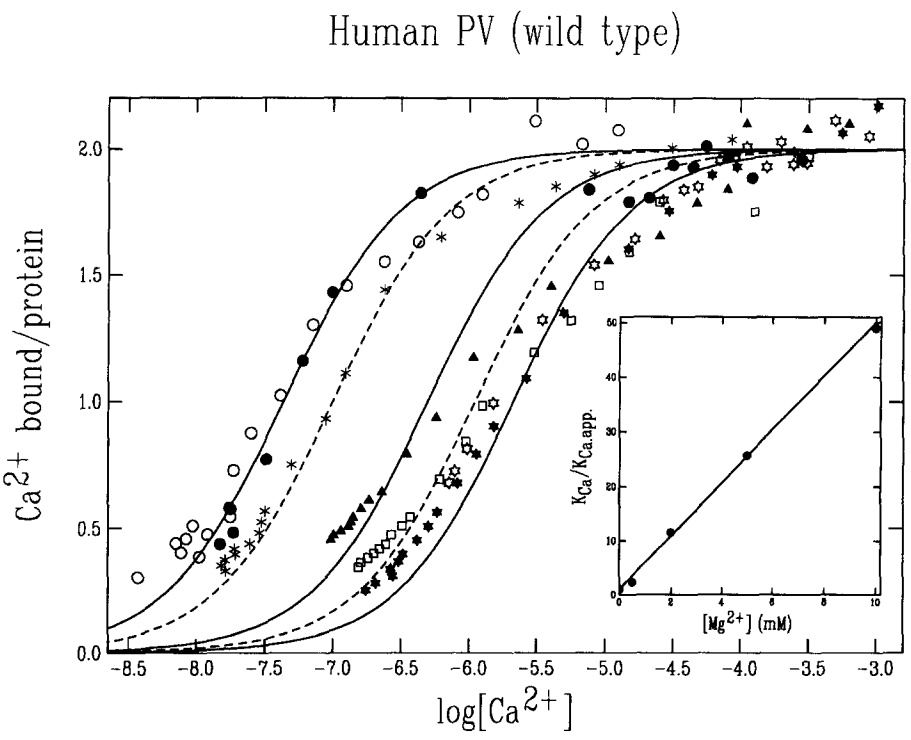

Fig. 2. Ca^{2+} binding to recombinant human PV_{wt}. Ca^{2+} binding was measured in solutions of 20 to 30 μM PV by the flow dialysis method at 25°C. Mg^{2+} concentrations were 0 mM (○, ●), 0.5 mM (*), 2 mM (▲), 5 mM (□), or 10 mM (☆, ★). The curves were calculated with the K_{ass} constants listed in Table 1. Inset: ratio of the intrinsic Ca^{2+}-binding constant in the absence of Mg^{2+} over the apparent Ca^{2+}-binding constant in the presence of Mg^{2+} as a function of $[Mg^{2+}]$.

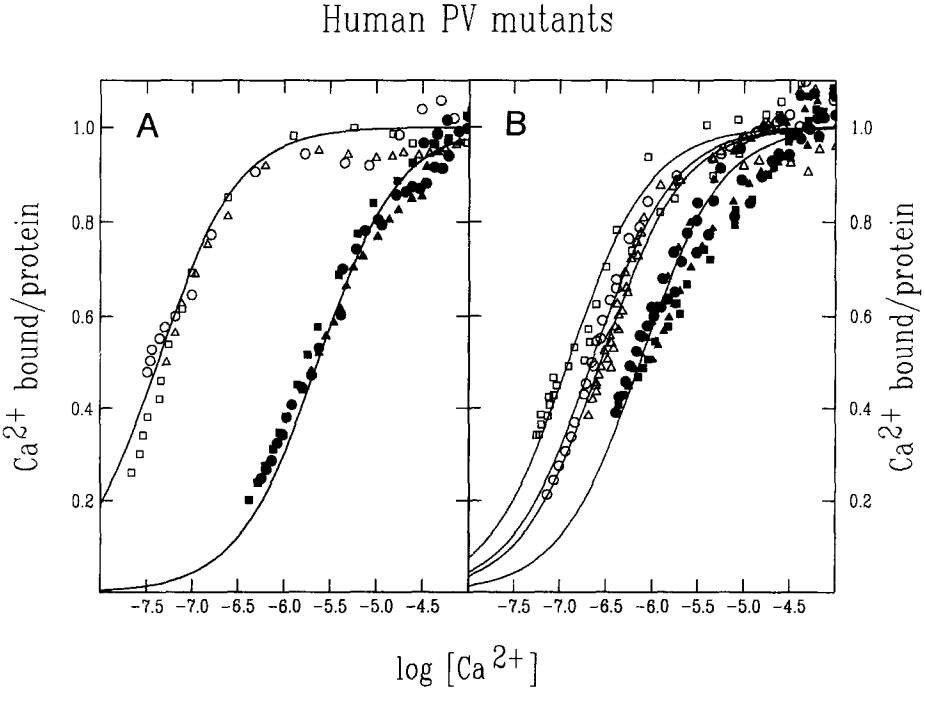

Fig. 3. Ca^{2+} binding to recombinant PV mutants with inactivated CD (A) or EF sites (B), respectively. The PV concentrations were 20 to 30 μM. Mg^{2+} concentrations were 0 (open symbols) or 10 mM (closed symbols). (A) ○,●, PV_{E62V}; □,■, PV_{D51A}; △,▲, $PV_{D51A,E62,V}$. (B) ○,●, PV_{E101V}; □,■, PV_{D90A}; △,▲, $PV_{D90A,E101V}$. The curves were calculated with the $K_{ass.}$ constants listed in Table 2.

matography. Recombinant proteins were homogeneous as judged by Coomassie brilliant blue staining of SDS-PAGE (Fig. 1B). Routinely, the yield of purified proteins was 50 to 70 mg/l of bacterial culture.

Analysis of the isoelectric points of each mutant protein was carried out under denaturing conditions (summarised in Table 1). PV_{wt} was found to be the most acidic protein, at pH 5.34, followed by the group of mutants, PV_{E101V}, PV_{D90A}, PV_{E62V}, and PV_{D51A}, around pH 5.45, mutants $PV_{D90A,E101V}$, $PV_{E62V,E101V}$, $PV_{D51A,D90A}$, and $PV_{D51A,E62V}$, around pH 5.67, and $PV_{D51A,E62V,D90A,E101V}$, at pH 6.32. The measured isoelectric points of the proteins were generally more basic when compared to the calculated values. The more acidic protein bands shifted by 0.18 pH units represent the *N*-acetylated proteins (see below).

3.2. ESI-mass spectrometry

To assure the correct sequence of the PV_{wt} and of the nine mutants, their molecular mass was accurately determined by ESI-MS. The molecular weights of all proteins were found to be in good agreement with the calculated values (Table 1). In all mutants a second protein species was detected with a mass increase of 42 Da, corresponding to N-terminal acetylated protein.

3.3. Direct binding studies

Fig. 2 shows the Ca^{2+}-binding isotherms of PV_{wt} in the presence of 0, 0.5, 2, 5, and 10 mM Mg^{2+} as measured by the flow dialysis method. In the absence of Mg^{2+} the intrinsic affinity constants of the two sites for Ca^{2+} are identical and equal to 2.3×10^7 M^{-1} – i.e., slightly lower than that of recombinant rat PV [17]. In increasing Mg^{2+} concentrations the isotherms are parallel-shifted to higher free Ca^{2+} concentrations, suggestive of strict competition at both sites. The $Ca_{0.5}$ values obey the competition equation (see Section 2) with $K_{Mg.compet.}$ equal to 4.9×10^3 M^{-1} (Fig. 2, inset).

In comparison with PV_{wt}, Fig. 3 shows the Ca^{2+}-binding isotherms of the mutant PVs with a single inactivated site in the presence of 0 and 10 mM Mg^{2+}. As predicted from the sequence, they display only one site for Ca^{2+}, without indications for an additional low-affinity site. The PV_{-CD} (PV_{E62V}, PV_{D51A}, and $PV_{D51A,62V}$) mutants display Ca^{2+}- and Mg^{2+}-binding properties to the EF site which are nearly independent of the way the CD site is inactivated (Fig. 3A). In the absence of Mg^{2+} they display K'_{Ca} values of 2.0 to 2.3×10^7 M^{-1}; in the presence of 10 mM Mg^{2+} the $K'_{Ca.app.}$ values equal 4.3 to 5.7×10^5 M^{-1}. Assuming straight competition between Ca^{2+} and Mg^{2+}, the $K'_{Mg.compet.}$ values are 3.4 to 4.6×10^3 M^{-1}. All these values are very similar to those of PV_{wt}, suggesting that the affinity of the EF site for Ca^{2+} and Mg^{2+} is not influenced by modifications in the CD site. The situation is quite different in the PV_{-EF} mutants (PV_{E101V}, PV_{D90A}, and $PV_{D90A,E101V}$), where each of the three modifications in the EF site leads to a substantial decrease of the affinity of the remaining CD site for Ca^{2+} as well as for Mg^{2+} (Fig. 3B). Interestingly, the Ca^{2+}-affinity depends on how the EF site is inactivated (Table 2): the modification in the −Z position of the EF site is more deleterious for the affinity than inactivation of the +X position, and inactivation of both residues yields a cummulative effect. 10 mM Mg^{2+} weakly affects the affinity of the CD site for Ca^{2+} with calculated $K'_{Mg.compet.}$ values of 1.7 to 4.7×10^2 M^{-1} (Table 2). As expected, the proteins $PV_{E62V,E101V}$, $PV_{D51A,D90A}$, and $PV_{D51A,E62V,D90A,E101V}$ with the two sites inactivated do not bind Ca^{2+} under the experimental conditions described above.

3.4. Fluorescence characteristics

After excitation at 259 nm, metal-free human PV_{wt} shows an emission spectrum with a maximum at 283 nm. Upon denaturation by 3 M guanidine HCl the fluorescence *in*creases 1.2-fold, whereas upon binding of either Ca^{2+} or Mg^{2+} the fluorescence *de*creases 1.8-fold (Fig. 4). PV_{D90A}, PV_{E101V},and $PV_{E101V,D90A}$ mutants show a behavior very similar to PV_{wt}. The mutant proteins PV_{E62V}, PV_{D51A}, and $PV_{D51A,62V}$ show a denaturation-induced 1.2-fold *in*crease in fluorescence and a small 1.1- to 1.2-fold *de*crease upon binding of Ca^{2+} or Mg^{2+}. Finally, $PV_{E62V,E101V}$, $PV_{D51A,D90A}$, and $PV_{D51A,E62V,D90A,E101V}$ show only a denaturation induced 1.2-fold increase in Phe fluorescence.

Table 2
Intrinsic association constants (K') of human PV_{wt} and nine inactivation mutants for Ca^{2+} and calculated K' for Mg^{2+} assuming straight competition

Intrinsic Ca^{2+} binding constants (K'_{Ca}) at varying Mg^{2+} concentrations			
PV_{wt}			
Mg^{2+} (mM)	PV_{wt}	K'_{Ca} / $K'_{Ca.app.}$	
0	2.3×10^7		
0.5	1.0×10^7	2.3	
2	2.0×10^6	11.5	
5	9.0×10^5	25.6	
10	4.7×10^5	48.9	$\Rightarrow K'_{Mg.compet.} = 4.9\times10^3$
PV_{-CD}			
Mg^{2+} (mM)	PV_{E62V}	PV_{D51A}	$PV_{D51A,E62V}$
0	2.3×10^7	2.0×10^7	2.0×10^7
10	5.0×10^5	5.7×10^5	4.3×10^5
$K_{Mg.compet.}$	4.5×10^3	3.4×10^3	4.6×10^3
PV_{-EF}			
Mg^{2+} (mM)	PV_{E101V}	PV_{D90A}	$PV_{D90A,E101V}$
0	4.5×10^6	7.9×10^6	3.6×10^6
10	1.6×10^6	1.4×10^6	1.3×10^6
$K'_{Mg.compet.}$	1.8×10^2	4.7×10^2	1.7×10^2

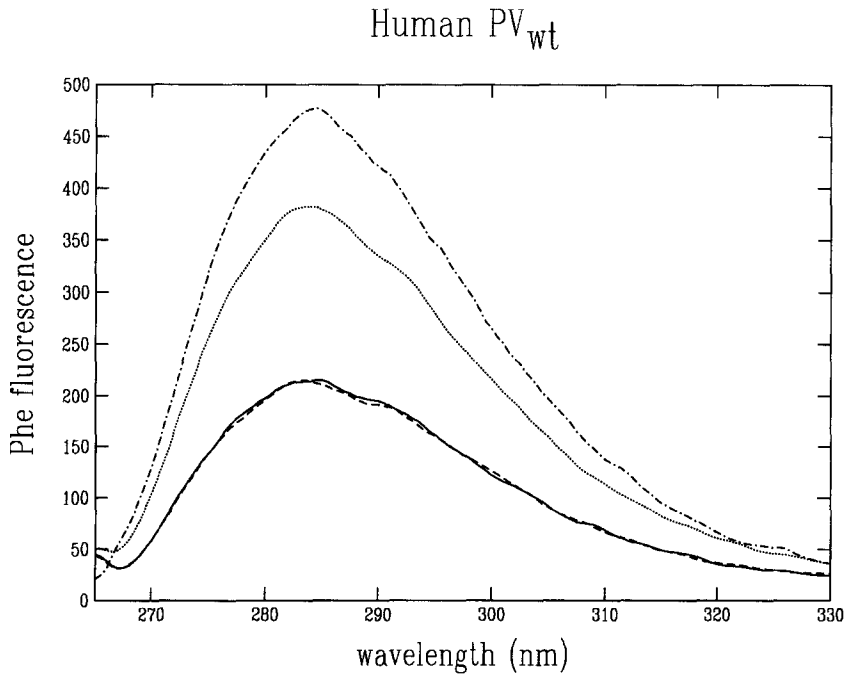

Fig. 4. Fluorescence spectra of PV_{wt}. The protein concentration was 50 μM. Ca^{2+}, Mg^{2+} or guanidine hydrochloride were added up to 1 mM, 2 mM, and 4 M, respectively. The spectra were normalized as described in Section 2. Solid line, Ca^{2+} form; dashed line, Mg^{2+} form; dotted line, metal-free form; dashed and dotted line, guanidine hydrochloride-denatured form.

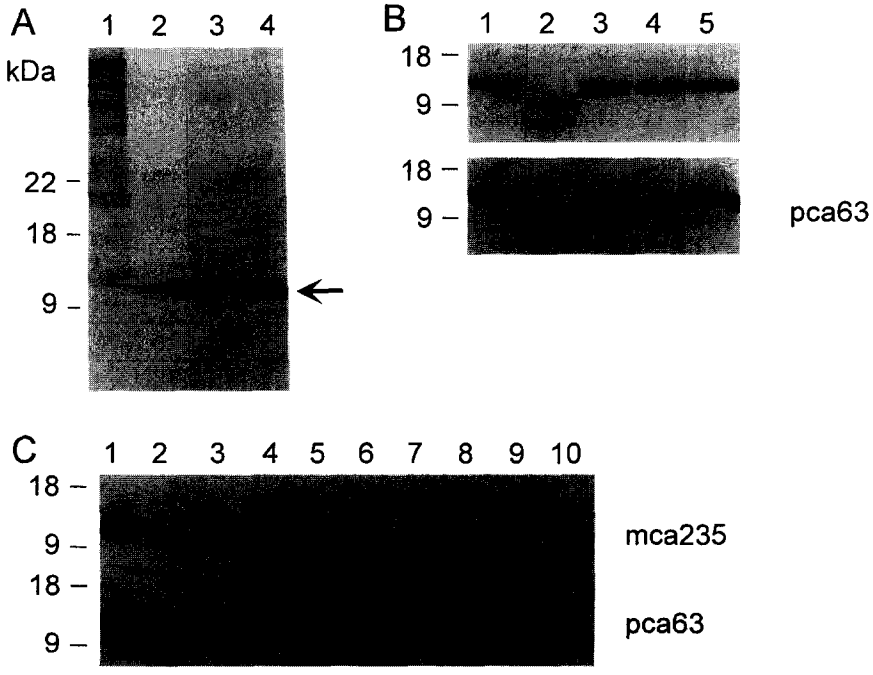

Fig. 5. Characterization of antibodies by Western blot analysis. (A) Human cerebellar protein extract (100 μg, lanes 1 and 3) and recombinant hPV_{wt} (lane 2, 2 μg; lane 4, 100 ng). Proteins were visualized by Coomassie blue (lanes 1 and 2), and detected on Western blot analysis using the pca63 serum (lanes 3 and 4). Arrow, position of PV (m.wt. 12 kDa). (B) Cross-reactivity of the human antiserum (pca63) with parvalbumins of other species. Top: SDS-PAGE stained with Coomassie brilliant blue of muscle PVs from (200 ng each PV) gerbil (lane 1); cat (lane 2); mouse (lane 3); rat (lane 4); PV_{wt} (lane 5). Bottom: corresponding Western blot with the pca63 serum. (C) immunoreactivity of the monoclonal anti-carp PV antibody mca235 (top) and of the polyclonal anti-human PV serum (pca63; bottom) against human PV_{wt} and the nine mutants (lanes 1–10; order of the samples see Fig. 1B) by Western blot analysis. 100 ng of each protein was applied.

3.5. Characterization of the specific polyclonal antibodies against human recombinant α-parvalbumin

The specificity of the antiserum pca63 was tested on Western blots against human cerebellum extract (Fig. 5A), against parvalbumins from other species (Fig. 5B), and against all nine mutant proteins (Fig. 5C). A specific immunoreaction for native human brain PV (Fig. 5A, lane 3) and recombinant human PV_{wt} (lane 4) was observed, demonstrating immunological identity. Strong cross-reactivity of pca63 with muscle α-PV from gerbil, cat, mouse, and rat was found and pca63 can therefore be used in these species for anatomical, developmental, and functional studies.

For the monoclonal antibody mca235 directed against carp muscle PV a specific epitope was mapped to lysin-54 located in the CD-loops of carp muscle parvalbumin (isoform with $pI = 4.25$) [20]. The mca235 was therefore used to test the consequences of the different mutations on the reactivity toward the antigenic CD-site (Fig. 5C, top). The antibody reacted strongly with PV_{wt} (lane 1) and PV_{-EF} (lanes 2, 3, 6), whereas a faint or no signal was detected with PV_{-CD} (lanes 4, 5, 9) and PV_{-CD-EF} (lanes 7, 8, 10), confirming the previous results. Our polyclonal antibody pca63 shows a similar reactivity (Fig. 5C, bottom), reacting stronger with the mutants PV_{-EF} than with the PV_{-CD} mutants, indicating that pca63 recognizes the same epitope as mca235. The additional but decreased reactivity against the mutants $PV_{-CD,-EF}$ (lanes 7, 8, and 10) indicates that additional epitopes also in the EF-loop are probably recognized.

These specific and well defined human antibodies can now be applied for localization and functional studies in human tissues and to monitor pathological changes, e.g., in human neurodegenerative disorders.

4. Discussion

In the present study nine mutant human parvalbumins containing either one or two abortive Ca^{2+}-binding sites in their C-terminal tandem domain have been analyzed. The highly conserved positions 1 (Asp) and/or 12 (Glu) in the 12 residue Ca^{2+}-binding loop which provide essential oxygen ligands for Ca^{2+} were replaced by the nonpolar residues Ala and Val, respectively. NMR showed that in the loops of pike PV these residues exhibit the largest changes upon metal binding [21]. The same replacements in all three functional loops in yeast calmodulin resulted in weak Ca^{2+}-binding when position 12 was changed, whereas no Ca^{2+}-binding was detectable when positions 1 and 12 were substituted [22]. Replacements of position 1, D → A, alone also prevented the binding of Ca^{2+} to yeast mutant calmodulins, whereas the same replacement for single sites in troponin C was not enough to inactivate the mutated site [23]. Our data revealed that in human PV the mutation of a single one of these critical residues is sufficient to inactivate cation binding to the site.

One of the most intriguing observations of our study is that, despite the structural symmetry in the 2-sites domain of parvalbumin, these two sites do not have the same importance within the paired EF-hand domain. In the recombinant protein the two sites appear to be equivalent with the same affinity for Ca^{2+} and the same antagonistic effect of Mg^{2+} on Ca^{2+}-binding. However, important differences are observed when either the N-terminal or the C-terminal site is modified so that it can no longer bind Ca^{2+}. When the CD site is inactivated, the remaining EF site has still the same high affinity for Ca^{2+} as the parent PV_{wt}. When the EF site is impaired the CD site binds cations with lower affinity. Apparently, in the paired domain the EF site is structurally much more important than the CD site and constitutes the stable structural nucleus of the paired domain. In addition, we show that the way of inactivation can influence the affinity of the remaining active site for Ca^{2+}: the inactivation of the CD site is less critical than the inactivation of the EF site: the mutation of Asp-90 to Ala decreases the affinity of the remaining CD site 3.6-fold, of Glu-101 to Val 5.1-fold, and the mutation of both residues yields a 6.4-fold decrease. The destabilization of the EF site seems to be gradually changing and cummulative.

The functional importance of parvalbumin as a buffer of Ca^{2+} and Mg^{2+} can now be evaluated by transfection of these mutant PVs in living neuronal cells where a model can be established that also holds true for human cells. The PV_{-CD} and PV_{wt} are supposed to be fully saturated with Mg^{2+} at rest, whereas PV_{-EF} would be mostly in a metal-free state. Therefore, upon increase of intracellular Ca^{2+} levels the former two proteins would first have to release their bound Mg^{2+} in order to take up Ca^{2+}, whereas PV_{-EF} could immediately bind Ca^{2+}. This allows to study separately the effect of Ca^{2+}/Mg^{2+} exchange and of Ca^{2+} buffering. The mutant $PV_{-CD/-EF}$ can yield indications whether the role of parvalbumin is restricted to the two above actions or includes also divalent cation-independent functions.

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